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Methods and Probiotics for Improving Starch Utilisation and Preventing Over-production of Acid in the Rumen or Gut of Animals

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The following statement is a full description of this invention, including the best method of performing it know to me:

Abstract

Methods and Probiotics for Improving Starch Utilisation and Preventing Over-production of Acid in the Rumen or Gut of Animals

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Methods for improving the utilisation of starch and preventing the over production of acid in an animal comprising administering to the animal probiotics including starch-utilizing bacteria and/or lactic acid-utilizing bacteria. The utilisation of starch is the utilisation of starch contained in diets such as grain based diets, and the over production of acid is acidosis including lactic acidosis in a ruminant and a monogastric animal. The monogastric animal is a horse and the ruminant is selected from the group consisting of sheep, cattle and goat. The improving the utilisation of starch is to ferment the starch to other end products rather than lactic acid by the starch-utilizing bacteria (*Ruminobacter, Prevotella, Bacteroides, Succinimonas, Butyrivibrio, Succinivibrio, Clostridium*, and other starch-utilizing bacteria, which produce no or a low level of lactic acid during the fermentation of starch). The prevention of over-production of lactic acid is to utilise lactic acid by the lactic acid-utilizing bacteria (*Megasphera, Peptococcus asaccharolyticus, Veillonella, Selenomonas, Anaerovibrio, and Propionibacterium*, and other microorganisms that are efficient at fermenting lactate). A combination of the starch- and lactic acid-utilizing bacteria is more effective than either the starch- or lactic acid-utilizing bacteria alone. About 100 strains including Su102, Su106, Su107, Su108, Su109, Su110, Su111, Su112, Su113, Su114, Su115, Su117, Su118, Lu12, Lu15, Lu21, Lu22, Lu23, and Lu24 were isolated and tested. Most of these strains belong to different genera of starch-fermenting or lactate-utilizing bacteria as mentioned above. The most preferred strains are Su109 (*Shuia liuiae* Su109), Su111 (*Prevotella shuii* Su111), and Lu12 (*Selenomonas ruminatium* subsp. *lactilytica* Lu12), which were newly isolated from the rumen fluid of a cattle at Grafton, Australia by the present inventors. The cattle had large numbers of Su109, Su111, and Lu12 in its rumen fluid, and did not show clinical signs of lactic acidosis after challenged with 90% grain. The probiotics may contain whole cells, cell lysatse, enzymes, or any part of the cells from the microorganisms, administering by the route of intraruminal, intragut, and/or oral. The probiotics include pharmaceutically or commercially acceptable carriers or excipients.

Methods and Probiotics for Improving the Utilisation of Starch and Preventing the Over-production of Acid in the Rumen or Gut of Animals

Technical Field

The present invention relates to new methods and new probiotics for improving the starch utilisation and preventing the over-production of acid in the rumen or gut of animals. In particular it is directed to methods and probiotics for alleviating acidosis and improving utilisation of starch in ruminants.

Background Art

The over production of acid in an animal by microorganisms can cause acidosis that is defined as a condition of pathologically high acidity of the blood. In ruminants the term is expanded to include acidic conditions in the rumen.

Acidosis in ruminants is a frequently observed acute condition that is also known as "grain engorgement". It occurs when the diet of ruminants is changed abruptly to contain large amounts of starch or other rapidly fermentable carbohydrates. A high incidence of acidosis is associated with feed lot livestock when their diet is rapidly changed from a forage-based ration to a grain-based ration. The rumen undergoes a marked decrease of pH initiated by increasing the rate in production of acids during the fermentative digestion of grains. The acids are then absorbed into the blood stream resulting in many of the clinical symptoms of acidosis. In many instances, *Streptococcus bovis* and *Lactobacillus* spp. become the dominant bacteria as pH drops resulting in the production of lactic acid which is then absorbed from the rumen. The growth and metabolic activity of these lactic acid producing organisms further lowers rumen pH. The condition of acidosis can be acute, posing a life-threatening situation, or chronic (sub-acute), resulting in reduced feed intake and weight gain.

Gross symptoms of acidosis include reduction or cessation of feed consumption (anorexia), loose faeces or diarrhoea, a listless, depressed or distressed appearance, founder or sore feet and death. Other symptoms that can be measured or observed after the onset of acidosis include decreased rate of grain and feed efficiency, high incidence of abscessed livers at slaughter, rumenitis in slaughtered or dead animals, altered blood metabolic profile and incidence of polioencephalomalacia.

Non-ruminant animals such as horses and the like are also susceptible to acidosis. The increasing popularity of horse-related sports and activities has also led to an increase in the total incidence of acidosis in these animals. Horses are often housed in close quarters and are routinely grain fed when other fodder is unavailable, leading to the increased risk of acidosis.

Acidosis can also be a major problem during drought conditions when grain is often the only food source available to livestock. To place animals on a pure grain diet under these harsh conditions can cause a high incidence of acidosis often leading to death. There is a real need to have an economical and efficient means of protecting animals from the incidence of the over production of acid and acidosis.

In order to minimise or prevent the incidence of acidosis in livestock, current practices centre around management techniques based on introducing grain gradually to animals. However, the procedure is time-consuming and expensive because it involves numerous changes to the diet, with frequent feeding and close monitoring of daily feed intake and it also reduces potential liveweight gain. Even with a gradual introduction, the diets must be carefully formulated and the risk of lactic acidosis and poor animal performance remains. Dietary buffers and several antibiotics have been studied and used in conjunction with management practices to control acidosis. Unfortunately, none of these approaches is considered totally satisfactory for application to the livestock industry. For example, some antibiotics are effective in preventing lactic acidosis by reducing the population of *S. bovis* and *Lactobacillus* spp. thereby controlling the over production of lactic acid. However, the use of feed antibiotics may be restricted due to the potential emergence of drug-resistant microbial strains and the risk of antibiotic residues in animal products. The presence of antibiotics in animal products may also endanger export earnings as many countries prohibit the importation of animal products containing antibiotics.

Immunisation, which has been developed by our group (PCT/AU96/00143), is able to induce salivary antibody responses against *S. bovis* and *Lactobacillus* spp., and thus reduce over production of lactic acid in the rumen when an animal is fed grain-based diets containing high levels of starch.

The present inventors have further developed an alternative strategy for preventing over production of acid and acidosis in animals by improving the utilisation of starch and reducing lactic acid accumulation.

Disclosure of the Invention

Accordingly, in a first aspect the present invention consists of methods, for improving the utilisation of starch and preventing the over production of acid in an animal. This involves administering, to the said animal, an amount of probiotics comprised of starch-utilizing and/or acid-utilizing microorganisms.

In a preferred embodiment of the first aspect, the utilisation of starch is the utilisation of the starch that is contained in all sorts of feeds, and more specifically is the utilisation of starch in diets such as grain-based diets. The over production of acid is acidosis, and more specifically lactic acidosis.

In a further preferred embodiment of the first aspect, the starch-utilizing microorganisms are the bacteria, which can utilise starch as an energy source and form part of the normal rumen or gut flora of an animal. The preferred choice of microorganisms are *Ruminobacter, Prevotella, Bacteroides, Succinimonas, Butyrivibrio, Succinivibrio,* and other starch-utilizing bacteria, which do not produce or produce a low level of lactic acid during the fermentation of starch. The preferred starch-utilizing strain is Su109-(*Shuia liuiae* Su109) and/or Su111 (*Prevotella shuii* Su111), which are newly isolated by the present inventors (see example 8). The acid-utilizing microorganisms are the bacteria, which can utilise lactic acid (or lactate) as an energy source and form part of

the normal rumen or gut flora of an animal. Preferably the microorganisms are lactic acid-utilizing microorganisms. More preferably the microorganisms are *Megasphera, Peptococcus (asaccharolyticus), Veillonella, Selenomonas, Anaerovibrio, and Propionibacterium* bacteria. The preferred lactic acid-utilizing strain is Lu12 (*Selenomonas ruminatium* subsp. *lactilytica* Lu12), which is newly isolated by the present inventors (see example 8).

In a still further preferred embodiment of the first aspect, the animal is a monogastric animal or a ruminant and more preferably the monogastric animal is a horse and the ruminant is selected from the group consisting of sheep, cattle and goats.

Administration of the probiotics may be intraruminal, intragut, and/or oral.

In a second aspect the present invention consists of probiotics that improve utilisation of starch and prevents the over production of acid in an animal comprising starch-utilizing and/or acid-utilizing microorganisms thereof.

In a preferred embodiment of the second aspect the over production of acid is acidosis, and more specifically is lactic acidosis.

In a still further preferred embodiment of the second aspect, the animal is a monogastric animal or a ruminant and more preferably the monogastric animal is a horse and the ruminant is selected from the group consisting of sheep, cattle and goats.

In a still further preferred embodiment of the first aspect, the starch-utilizing microorganisms are the bacteria, which can utilise starch as an energy source and form part of the normal rumen or gut flora of an animal. Preferably the microorganisms are *Ruminobacter, Prevotella, Bacteroides, Succinimonas, Butyrivibrio, Succinivibrio, Clostridium* and other starch-utilizing bacteria, which produce no or a low level of lactic acid during the fermentation of starch. The preferred starch-utilizing strain is Su109 and/or Su111. The acid-utilizing microorganisms are the bacteria, which can utilise lactic acid (or lactate) as an energy source and form part of the normal rumen or gut flora of an animal. Preferably the microorganisms are lactic acid-utilizing microorganisms. More preferably the microorganisms are *Megasphera, Peptococcus asaccharolyticus, Veillonella, Selenomonas, Anaerovibrio, and Propionibacterium* bacteria. The preferred lactic acid-utilizing strain is Lu12.

The probiotics may comprise the starch-utilizing and/or lactic acid-utilizing bacteria whole cells, cell lysate, enzyme, or any part of the cells. The probiotics may also contain pharmaceutically or commercially acceptable carriers or excipients known to the art.

In a third aspect the present invention consists in the bacterial strain Lu12, Su109, and Su111, deposited with the Australian Government Analytical Laboratories (AGAL) and given accession number NM99/09083 (Lu12), NM99/09084 (Su109), and NM99/09085 (Su111).

In order that the nature of the present invention may be more clearly understood, preferred forms thereof will be described with reference to the following examples.

Modes for Carrying out the Invention

Example 1: Significant increase of the number of starch-utilizing and lactic acid-utilizing bacteria in animals after feeding diets rich in starch

In response to a diet, which is rich in starch, starch- and lactic acid-utilizing bacteria in the rumen or gut of animals may significantly increase in numbers to ferment the starch and reduce the lactic acid accumulation. This example shows the increase of starch-utilizing and lactic acid-utilizing bacteria in the rumen of animals.

Materials and Methods

Four Merino wethers grazing dry native pasture were selected and penned individually, feeding chaffed lucerne hay (800 g/day). Two of the sheep (sheep 3 and 4) remained on the chaffed lucerne hay diet until the end of the experiment, while the other two sheep (sheep 1 and 2) were adapted to a diet consisting of 75% wheat plus 25% chaffed lucerne hay (800 g/day). After 3 months, the two sheep were changed to 1 kg/day of a diet consisting of 50% wheat plus 50% chaffed lucerne hay.

Samples of rumen fluid were taken from sheep on chaffed lucerne hay, after 1 month on the 75% wheat diet, and after 1 month on the 50% wheat diet. When the liveweight of the sheep was measured, rectal samples of faeces were also taken. These were used to measure rumen and faecal pH, rumen lactate, volatile fatty acids (VFA) and bacteria population. The number of lactic acid-utilizing bacteria was measured by using LH medium (Mackie and Gilchrist 1979). The numbers of starch-utilising bacteria were measured using modified RF^{+} (mRF) (Shu, 1997) agar. RF^{+} agar was used for enumerating the total bacteria numbers. The mBA and MRS media were used for measuring the *S. bovis* and *Lactobacillus* spp. (Shu, 1997).

Results and Conclusions

The numbers of starch- and lactic acid-utilizing bacteria in the rumen fluid of the sheep on grain diets were significantly higher than the number in the rumen fluid of the sheep on lucerne hay (Table 1). The ratios of *S. bovis* and *Lactobacillus* spp. to the total starch-utilizing bacteria increased after adapted to the grain diets (data not shown).

Table 1 Changes of lactate- and starch-utilising bacteria and total bacteria numbers in rumen fluid of sheep (shown in Log number of CFU)

	Chaffed lucerne hay	75% Wheat	50% Wheat
Starch-utilizing bacteria	5.87±0.10	9.31±0.32**	8.58±0.13**
Lactic acid-utilizing bacteria	5.43±0.39	7.32±0.25*	6.50±0.27*
Total bacteria	9.40±0.19	10.0±0.11	9.45±0.15

Values with *($P<0.05$) and ** ($P<0.01$) are significantly different from that of chaffed lucerne hay in same row.

No clinical signs of acidosis were observed during the whole experimental period. The results of feed intake, liveweight gain, pH, etc. also show that the animals had adapted well to the grain diets and were healthy. Starch- and lactic acid-utilizing bacteria increased to a significantly high level after animals fully adapted to the grain diets. At the same time, the ratio of *S. bovis* or *Lactobacillus* spp. to total starch-utilizing bacteria

was maintained at a low level. This indicates that the higher proportion of starch-utilizing bacteria are not lactic acid-producing bacteria are *S. bovis* and *Lactobacillus* spp.. These results support the theory that artificially enhancing the numbers of starch-utilizing bacteria (which produce no or a low level of lactic acid) and/or lactic acid-utilizing bacteria may improve the utilisation of starch and prevent the over-production of lactic acid in the rumen or gut of an animal.

Example 2: Isolation of starch- and lactic acid-utilizing bacteria from sheep and cattle

This example demonstrates the isolation of 1) starch-utilizing bacteria, which do not produce or produce low level of lactic acid; and 2) lactic acid-utilizing bacteria strains, which are highly efficient in utilizing lactate.

Materials and Methods

Samples of rumen content, rumen fluid and faeces were collected from sheep and cattle in the University of New England Animal House, Kirby Animal House, Kirby Farm, Tullimba Feedlot Facility, and Grafton Agriculture Research Station, Australia. Samples were transported to the laboratory immediately, and diluted by the procedures described by Ogimoto and Imai (1981). Lactate-utilizing bacterial strains were isolated using the roll tube method described by Hungate (1969). The selective LH medium was used for the roll tube agar (Mackie and Heath, 1979). Starch-utilizing bacterial strains were isolated by the same method except using mRF.

The utilisation of lactic acid by the lactic acid-utilizing bacterial isolates was measured by using the following procedures. Each of the strains was cultured in MLH (modified LH) broth at 38.5°C for 20~24 hours. The number of bacterial cells in the culture was determined by direct microscopic counting and adjusted to 1×10^{10} cells/ml in MLH broth. A 0.10 ml aliquot of the culture was transferred into 5 ml of MLH broth and incubated for 24 hours; also a 0.10 ml aliquot of heat-killed culture was used as a control. After incubation, the broth was centrifuged at 10,000 g for 15 mins, and the supernatant was collected. The concentration of D- or L- lactate in the supernatant was analysed. The lactic acid producing capacities of the starch-utilizing bacteria strains were measured by using the above procedures except that mRF was used instead of MLH.

Results

About 100 isolates of lactate- and starch-utilizing bacteria were obtained. Lactic acid-utilizing bacterial isolate Lu12 utilised almost all of the lactate in the broth. Both L- and D-lactate were lower than 5.5 mmol/L in the broth after fermentation by Lu15. After fermentation by the other isolates, the lactate concentrations in the broth were still higher than 46 mmol/L. The isolates, which produced lactate, lower than 0.5 mmol/L, included Su102, Su106, Su 107, Su108, Su109, Su111, Su112, Su114, Su115, Su117, and Su118. Figure 1 and Table 3 demonstrate the results of some of the isolates.

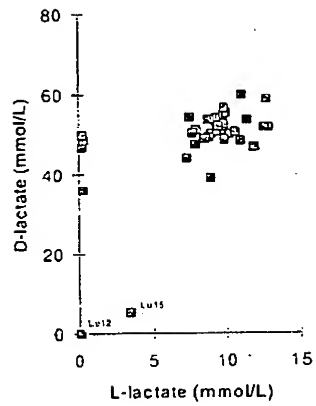


Figure 1 Lactate concentration (mmol/L) in MLH after incubation with lactic acid-utilizing bacteria

Table 3 Lactate production (mmol/L) in mRF by the starch-utilizing bacteria

Isolate	L-lactate		D-lactate	
	Mean	se	Mean	SE
Su102	0.12	0.05	0.04	0.02
Su103	0.05	0.01	29.12	1.50
Su104	0.07	0.02	36.31	5.31
Su105	0.04	0.03	22.15	1.18
Su106	0.06	0.02	0.04	0.05
Su107	0.01	0.01	0.02	0.02
Su108	0.01	0.02	0.02	0.01
Su109	0.07	0.01	0.06	0.04
Su110	0.11	0.06	1.99	1.22
Su111	0.31	0.12	0.15	0.13
Su112	0.01	0.01	0.03	0.01
Su113	0.00	0.03	1.87	1.00
Su114	0.07	0.06	0.19	0.18
Su115	0.04	0.02	0.02	0.02
Su117	0.11	0.11	0.09	0.07
Su118	0.06	0.06	0.19	0.19

Conclusions

About 100 strains of lactate- and starch-utilizing bacteria strains have been isolated from a wide range of samples. The lactate fermentation test demonstrated that Lu12 was the most efficient lactic acid-utilizing strain. Lu15 was the second most efficient strain. Lu21, Lu22, Lu23, and Lu24 can utilise most of the L-Lactate but not the D-lactate. These isolates belong to different genera including *Megasphera, Peptococcus, Veillonella, Selenomonas, Anaerovibrio, and Propionibacterium.* The isolates (Su102, Su106, Su107, Su108, Su109, Su111, Su112, Su114, Su115, Su117, and Su118) produce very low levels of lactic acid. These isolates belong to different genera including *Ruminobacter, Prevotella, Bacteroides, Succinimonas, Butyrivibrio, Succinivibrio,* and other starch-utilizing bacteria, which do not produce or produce low levels of lactic acid during the fermentation of starch.

Lu12, Su109 and Su111 were isolated from the rumen fluid of a cattle at Grafton Agriculture Research Station, Australia. The cattle had large numbers of these three strains in its rumen fluid, and did not show clinical signs of lactic acidosis after challenged with 90% grain diet.

Example 3: Utilisation of lactate in broth containing high concentrations of starch

There is a high level of starch in the rumen or gut fluid of an animal fed diets rich in starch. Glucose is a key intermediate of the starch metabolism. This example demonstrates the production of lactic acid by starch-utilizing bacteria, and the utilisation of lactate by lactic acid-utilizing bacteria in broth containing high levels of starch and glucose.

Materials and Methods

Broth: mRF-2, modified from mRF broth, containing 2% starch and 0.5%glucose; MLH-2: adding 0.2% bacteriological peptone, 1% hemin (0.05%) solution, and 1% VFA solution in MLH broth; MLH-3: adding 2% soluble starch and 0.5% D-glucose to MLH-2. *Starch-utilizing bacterial strains:* Su102, Su106, Su107, Su108, Su109, Su110, Su111, Su112, Su113, Su114, Su115, Su117, and Su118. *Lactic acid-producing bacterial strains:* Sb-5, LBT023, LBT029, LBT040, and LBT061. *Lactic acid-utilizing bacterial strains:* Lu12, Lu15, Lu21, Lu22, Lu23, and Lu24. Starch-utilizing bacterial strains were inoculated in mRF-2 broth, while the lactic acid-utilizing bacterial strains were inoculated in MLH-2 and MLH-3 broth, and then cultured for 24 hours. Lactate was measured after incubation.

Results

Each of the starch-utilizing bacterial strains (Su102, Su106, Su107, Su108, Su109, Su110, Su111, Su112, Su113, Su114, Su115, Su117, and Su118) produced no more than 2.90 mmol/L lactic acid, while any one strain of the lactic acid-producing bacteria *S. bovis* and *Lactobacillus* spp. (Sb-5, LBT023, LBT029, LBT040, LBT061) produced more

than 16.29 mmol/L lactic acid. The starch-utilizing bacterial strains did not grow in the MLH-2 broth.

In the MLH-2 broth, low levels of lactic acid were detected after fermentation by the lactic acid-utilizing bacterial strains. However, higher levels of lactate were found in MLH-3 broth after fermentation by the lactic acid-utilizing bacteria. For example, in MLH-2 there was no more than 0.72 mmol/L lactate after fermentation by Lu12 and Lu15; but in the MLH-3, the lactate levels were greater than 54.85 and 67.24 mmol/L respectively.

There was a significantly lower level of lactate in MLH-3 broth after fermentation by a combination of the starch- and lactic acid-utilizing bacterial strains. For example, in MLH-3 there were no more than 4.82 mmol/L lactate detected after fermentation by the combination of Lu12 and Su109 or Lu12 and Su111.

Conclusions

The starch-utilizing bacterial strains do not produce or produce very low levels of lactic acid in the presence of high levels of starch and glucose. The lactic acid-utilizing bacteria can utilise a high level of lactate when lactate is the only energy source. When starch and glucose are present a combination of starch- and lactic acid-utilizing strains is more effective than the lactic acid-utilizing bacteria alone.

Example 4 Improving starch utilisation and preventing lactic acid accumulation in the rumen fluid of sheep and cattle challenged with starch (I)

When a high level of starch is present in the rumen or gut fluid of an animal, the lactic acid-producing bacteria (*S. bovis* and *Lactobacillus* spp.) may overgrow and produce a high level of lactic acid, which leads to lactic acidosis. Artificially increasing the number of starch-utilizing bacteria, which produce no or a low level of lactic acid, in the rumen or gut fluid will improve the utilisation of starch by: 1) fermenting the starch to other end products rather than lactic acid; 2) competing for the starch with the lactic acid-producing bacteria resulting in less starch being fermented by the lactic acid-producing bacteria, thus producing less lactic acid. Artificially increasing the number of lactic acid-utilizing bacteria in the rumen or gut fluid will enhance the capacity of rumen or gut fluid to remove the excess lactic acid, preventing the over-production of lactic acid. This example demonstrates the improvement in starch-utilisation and prevention of lactic acid accumulation in the rumen fluid of sheep and cattle challenged with starch.

Materials and Methods

Ten sheep and ten cattle were penned individually on a maintenance roughage diet with free access to. Ten grams of soluble starch was dissolved in 100 ml sterile distilled water by heating and stirring. The 10% starch solution was maintained at 38.5°C in a water bath until use. This solution was further diluted to 4%, 6%, and 8% starch solutions and maintained at the same temperature in the water bath. To a 15 ml centrifuge tube, 1.80 ml of 4%, 6%, or 8% starch solutions was added and pre-warmed in

the 38.5°C water bath. 1.80 ml of sterile distilled water was used as a non-bacteria control. Rumen fluid of sheep and cattle was collected and put in the pre-warmed above tubes directly to 9 ml scale and mixed well (final starch concentration was 0.8%, 1.2%, 1.6%). An aliquot of each of the single bacterial strains or a combination of starch- and lactic acid-utilizing bacteria cultures was also added, and incubated at 38.5°C water bath for 20 hrs. The pH and lactate of the rumen fluid was measured at the end of incubation.

Results

After incubation, the pH in the rumen fluid treated with starch- and/or lactic acid-utilizing bacteria strains were higher than the control (Figure 2). All the values of pH in the groups treated with Lu12 and the combinations of Lu12 and the starch-utilizing bacteria isolates were higher than that in the control group ($P<0.01$). The mean values of pH in the groups treated with the combination of Lu12 and Su109 or Lu12 and Su111 was higher than that in the group treated with Lu12 alone ($P<0.05$).

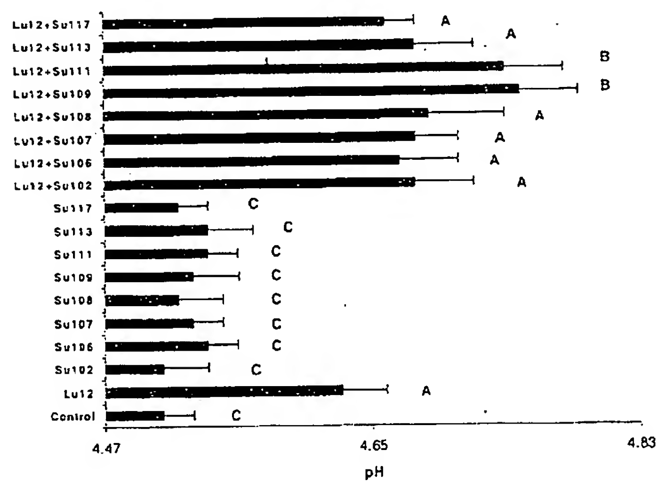
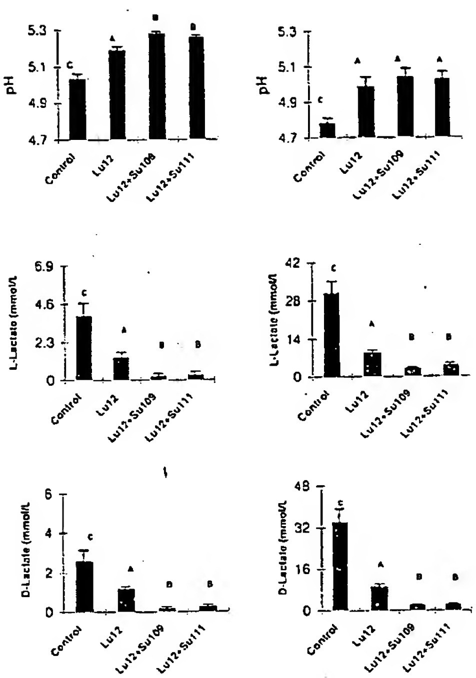


Figure 2 pH in the rumen fluid (1.6% starch) after incubated with either a single lactate- or starch-utilizing bacteria isolate or a combination of lactate- and starch-utilizing bacteria strains

Lu12 and Su109 or Su111 were used to prevent lactic acid accumulation in the rumen fluid challenged with either 0.8% or 1.2% starch. The values of pH, L-Lactate, and D-Lactate in the bacterial isolates treatment groups were significantly ($P<0.01$) higher than that in the controls (Figure 3).



(0.8% starch) (1.2% starch)

Figure 3 pH, L-lactate, D-lactate (mmol/L) in the rumen fluid (challenged by 0.8% or 1.2% starch) after incubation with Lu12, Su109, and Su111

The results obtained by using the cattle rumen fluid followed the similar patterns to those described above.

Conclusions

These results show that the starch- and/or lactic acid-utilizing bacterial strains can improve the starch utilisation and prevent the over-production of acid in the rumen fluid of sheep and cattle. A combination of the starch- and lactic acid- utilizing bacteria is more efficient than either the starch- or the lactic acid-utilizing bacterial strain alone. Lu12 plus either Su109 or Su111 was the most effective combination. It is worth noting that a significant difference in lactate concentration between groups Lu and Lu12+Su109 and Lu12+Su111 was observed ($P<0.05$), although the pH was at a similar level

(P>0.05). This result suggests that there are other acids such as VFA produced by the starch- and lactic acid- utilizing bacteria.

Example 5 Improving starch utilisation and preventing lactic acid accumulation in rumen fluid of sheep and cattle challenged with starch (II)

Lactic acid accumulation is due to the over-growth of lactic acid-producing bacteria (*S. bovis* and *Lactobacillus* spp.). Artificially increasing the number of starch- and lactic acid-utilizing bacteria in the rumen of gut fluid of animals will have two effects: 1) less starch will be available for *S. bovis* and *Lactobacillus* spp. growth; 2) maintaining a high level of pH will allow other microorganisms to survive and grow in the rumen fluid. As a result, the growth of *S. bovis* and *Lactobacillus* spp. may be inhibited. One of the main aims of this example is to compare the numbers of *S. bovis* and *Lactobacillus* spp. in the rumen fluid of sheep and cattle between treatment groups.

Materials and Methods

Eighteen sheep and 18 cattle were penned individually on a maintenance roughage diet with free access to. A total volume of 450 ml of rumen fluid from two sheep or cattle (225 ml each) was placed in a pre-warmed 500 ml Wheaton-bottles and mixed well. A total of 9 bottles (430 ml each) of rumen fluid were obtained and randomly allocated into 3 treatment groups on the basis of pH. Sixty millilitres of 10% starch solution and 10 ml of bacteria culture were then added each bottle (Table 4). The final starch concentration was 1.2%. All the bottles were mixed thoroughly, and the cap of each bottle was poled to release gas. The bottles were then incubated at 38.5° C in a water bath for 20 hours. pH, lactate, numbers of *S. bovis* and *Lactobacillus* spp., and VFA were measured.

Table 4 Treatment groups

	Treatment group		
	Control (n=3)	Lu12+Su111 (n=3)	Lu12+Su109+Su111 (n=3)
Rumen fluid of sheep or cattle (ml)	440	440	440
Starch solution (10%) (ml)	60	60	60
Live Lu12 culture (ml)	-	10	10
Live Su109 culture (ml)	-	-	10
Live Su111 culture (ml)	-	10	10
Killed Lu12 culture (ml)	10	-	-
Killed Su109 culture (ml)	10	10	-
Killed Su111 culture (ml)	10	-	-

Results

The pH in the rumen fluid incubated with lactate- and starch-utilizing bacteria was significantly higher than that found in the control (P<0.01). There was no difference in the pH between the groups treated with Lu12+Su111 and Lu12+Su109+Su111 (P>0.05). The L-Lactate concentration in the rumen fluid incubated with lactate- and starch-utilizing bacteria were significantly lower than that found in the control (P<0.01) (Figure

4). There was no difference in the L-Lactate concentration between the groups treated with Lu12+Su111 and Lu12+Su109+Su111 (P>0.05). The D-Lactate concentration in the rumen fluid incubated with lactate- and starch-utilizing bacteria was significantly lower than that in the control (P<0.01). There was no difference in the lactate concentration between the groups treated with Lu12+Su111 and Lu12+Su109+Su111 (P>0.05). Compared to the control, significantly higher VFA concentrations were found in the groups incubated with lactate- and starch- utilizing bacteria (P<0.01). There was no statistically significant difference between the groups of Lu12+Su111 and Lu12+Su109+Su111 (P>0.05).

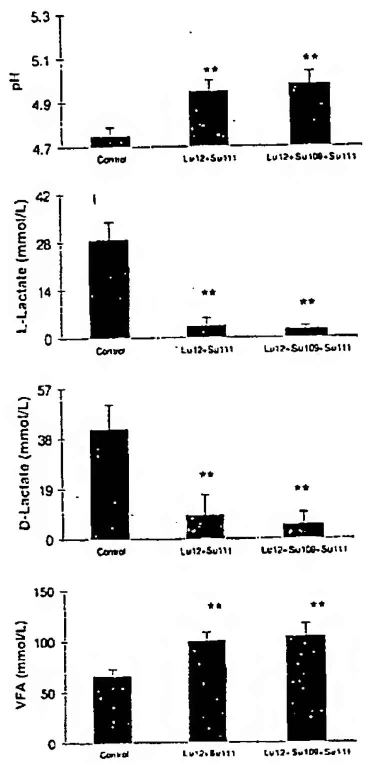


Figure 4 pH, lactate (mmol/L), and VFA (mmol/L) in the rumen fluid after incubation with the lactate- and starch-utilizing bacteria

The numbers of *S. bovis* and *Lactobacillus* spp. in the rumen fluid after incubation of the treatment groups are summarised in Figure 5. The numbers of *S. bovis* and *Lactobacillus* spp. in the groups incubated with lactate- and starch-utilizing bacteria were lower than in the control group (*$P<0.05$). No significant difference was found between the two groups incubated with the lactate- and starch-utilizing bacteria ($P>0.05$).

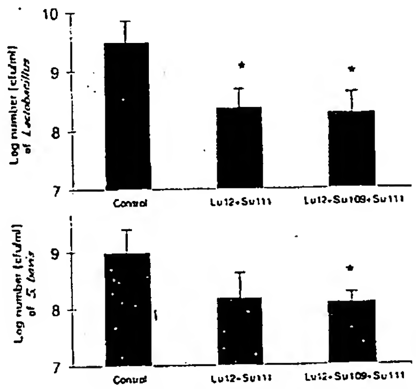


Figure 5 Number of *S. bovis* and *Lactobacillus* spp. in the rumen fluid after incubation with lactate- and starch- utilizing bacteria

The results obtained by using the cattle rumen fluid followed patterns similar to those described above.

Conclusions

Lower pH and lactate concentrations were found in the rumen fluid treated with the lactate- and starch-utilizing bacterial strains. These results provided further evidence to verify the observation that the accumulation of lactic acid in rumen fluid of sheep and cattle can reduced by lactate- and starch-utilizing bacteria. The results also indicate that the use of starch- and lactic acid-utilizing bacteria can inhibit the growth of *S. bovis* and *Lactobacillus* spp.. The mechanisms for the reduction of lactic acid accumulation in the rumen fluid treated with the lactate- and starch- utilizing bacteria may be mainly due to: 1) the starch-utilizing bacteria preferably ferment starch without producing lactic acid, 2) the lactic acid-utilizing bacteria remove the lactic acid produced by lactic acid-producing bacteria, and 3) the use of the bacteria inhibits the growth of lactic acid-producing bacteria (*S. bovis* and *Lactobacillus* spp.). The third aspect of the mechanism is important for controlling lactic acidosis, and is consistent with the mechanism of antibiotics and anti-acidosis vaccine (Tung and Kung, 1993; Thorniley *et al.*, 1996; Shu, 1997).

The present experiment clearly indicates that there were higher ($P<0.01$) levels of VFA in the rumen fluid incubated with the lactate- and starch-utilizing bacterial strains,

compared with that in the rumen fluid without adding the bacteria. This is because these bacterial strains have the ability to ferment starch to VFA without producing lactic acid. This provides further evidence to support that the starch- and lactic acid-utilizing bacteria can improve the utilization of starch and prevent the over-production of lactic acid in the rumen of sheep and cattle.

Another aim of this experiment was to test the effectiveness of the combinations of Lu12+Su109+Su111 and Lu12+Su111. There were no differences in pH, lactate, numbers of *S. bovis* and *Lactobacillus* spp., and the VFA concentration between those two groups. These results suggest that the combination of the three strains (Lu12+Su109+Su111) have a similar effect as the two strains (Lu12+Su111) when one starch is used. However, there are many types of grain starches, which may be used for feeding animals, and one starch- utilizing bacterial strain may have enzymes that are effective at degrading only certain types of starches. Therefore, the use of a combination of Lu12+Su109+Su111 would be more effective than Lu12+Su111 when several different starches are present. It will be useful to select certain strains or a combination of several strains to deal with a wider range of grain feeding.

Example 6 Improving starch utilisation and preventing acidosis in animals challenged with 50% grain

This example shows the effectiveness of the starch- and lactic acid-utilizing bacteria on animals challenged with 50% grain. The effectiveness was assessed by measuring the feed intake, liveweight, blood PCV, rumen pH, lactate and VFA, faecal pH and dry matter content.

Materials and Methods

Eighteen first cross wethers (around 3~4 years old) were used for the experiment. The sheep had been previously cannulated and penned individually on a maintenance roughage diet (80% oaten chaff plus 20% lucerne chaff) for 4 to 6 months. They were checked for any physical abnormalities before starting the experiment. All animals were fed an introductory diet consisting of approximately 1,200 g/day/sheep of oaten chaff (containing 1% urea) for 14 days, then 1,500 g/d/sheep of the oaten chaff was offered for another 7 days. Animals were randomly allocated to 5 treatment groups on the basis of liveweight and feed intake on roughage diet (Table 5). Then, the animals were inoculated with the lactate- and starch-utilizing bacteria cultures directly into the rumen and introduced to a 50% grain diet (50% wheat and 50% roughage), except for group 5 which was used as a non-grain feeding control.

Feed intakes (wheat, oaten chaff, and total intake) were measured daily (Shu, 1997). Animals were weighed before and after grain feeding to measure liveweight changes in the different treatment groups. Samples of blood were taken from all the sheep through jugular venipuncture before and after the grain challenge for measuring PCV (packed cells volume). Rumen fluid and faeces were collected on Days -3, 1, 3, and

10. Samples of rumen fluid were taken for measuring pH, lactate and VFA. Approximately 3g of faeces were mixed well with 3 times distilled water, immediately after sampling, for measuring faecal pH. Faeces were also collected for measuring faecal dry matter content.

Table 5 Feeding regimen and treatment groups

Day	Group				
	Control (n=4)	Lu (n=4)	Su (n=4)	Lu+Su (n=4)	Chaff (n=3)
-21--1	D1	D1	D1	D1	D1
0	D2	D2+BP1	D2+BP2	D2+BP3	D1+BP3
0-11	D2	D2	D2	D2	D1

D1, Roughage diet (oaten chaff containing 1% urea); D2, Grain diet (50% roughage + 50% wheat diet). BP1, 100ml of Lu12 culture. BP2, 150ml of starch-utilizing bacteria culture: 100ml Su 111 and 50ml Su 10. BP3, 250ml of combination of lactate- and starch- utilizing bacteria cultures: a) 100ml Lu 12; b) 100ml Su 111, and c) 50ml Su 109 cultures. The concentrations of the bacteria cells were 7.00×10^9 /ml (Lu12), 2.25×10^9 /ml (Su109), and 9.40×10^{10}/ml (Su111).

The composition of the media is:

Medium for growth lactate-utilizing isolates (LAM): 20 ml Distilled water, 20 ml DL-Lactic acid (Sigma, sodium salt) (10% solution), 2.0 g Trypticase peptone (BBL), 0.2 g Bacteriological peptone, 0.2 g Yeast extract, 7.5 ml Mineral solution I, 7.5 ml Mineral solution II, 40.0 ml Rumen fluid, 7.0 ml $NaHCO_3$ (9.1% solution), 50 mg Cysteine-HCl·H_2O (BDH). It was sterilised by autoclaving at 107°C for 45 minutes.

Medium for growing starch- utilizing bacteria (SM2): 40 ml Distilled water, 2 g Trypticase peptone, 0.2 g Peptone, 0.2 g Yeast extract, 7.5 ml Mineral solution I, 7.5 ml Mineral solution II, 40 ml Rumen fluid, 0.5% Soluble starch, 10 ml $NaHCO_3$ (9.1% solution), 50 mg Cysteine-HCl-H2O. It was sterilised by autoclaving at 107°C for 45 minutes.

Results

The overall wheat intake in both the Lu and Lu+Su groups were higher than the control ($P<0.05$). The growth rate in the animals treated with a combination of the lactate- and/or starch-utilizing bacteria was higher than the control. The combination of the starch- and lactic acid-utilizing bacteria had the highest growth rate (Figure 6). The PCV in the animals treated with a combination of lactate- and starch-utilizing bacteria changed little after the grain feeding (group Lu+Su) or no grain feeding (Chaff group) (Figure 7). The PCV change in the animals of the control group was significantly higher ($P<0.05$), compared to the animals in either Lu+Su or Chaff group.

Conclusions

These results indicate that the animals treated with the lactate- and/or starch-utilizing bacteria have higher feed intakes and liveweight gain, lower PCV changes. The intake and liveweight gain of the animals treated with the combination of lactate- and starch-utilizing bacteria was the greatest, while the PCV change was the lowest. The lower change in PCV indicates that there was less change in the blood water content. The higher increase of PCV in the control animals indicates that there was more blood water loss indicating that the animals were more seriously affected by lactic acidosis. These results support that the starch- and/or lactic acid-utilizing bacteria can improve the utilization of starch and reduce the risk of lactic acidosis. A combination of starch- and lactic acid-utilizing bacteria is more effective. This example indicates that the lactate- and starch-utilizing bacteria can remove the necessity to gradually introduce a ruminant

animal to 50% grain diet. The application of this approach would save huge labor and other related costs and also enhance animal production in the feedlot industry.

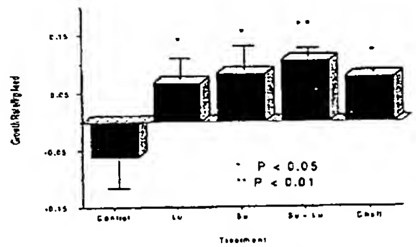


Figure 6 Growth rate of sheep. The growth rate in the Lu and/or Su groups was significantly higher than the control (P<0.05). The Lu+Su group had the highest growth rate.

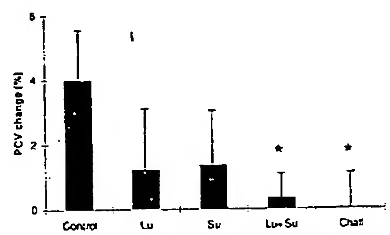


Figure 7 Changes of PCV in the sheep of different treatment groups. *The PCV changes in the Lu+Su and Chaff groups were significantly lower than the control (P<0.05).

Example 7 Improving starch utilisation and preventing acidosis in animals challenged with 75% grain

In the feedlot industry, the final ratios normally contain around 75% grain. This experiment further demonstrates the abilities of the starch- and lactic acid- utilizing bacteria for improving starch utilisation and reducing lactic acidosis in animals challenged with a 75% grain diet. Effectiveness was assessed by the number of animals withdrawn from 75% grain challenge, feed intake, liveweight gain, rumen pH, lactate, VFA, blood PCV, faecal pH and diarrhoea score.

Materials and Methods

Thirty-five Merino wethers were selected and penned individually, feeding chaffed oat hay (containing 1% urea, 800g/day/sheep) with free access to water at all times. After 19 days acclimatisation, 28 wethers were re-selected on the basis of liveweight, feed intake, healthy appearance and randomised into 4 treatment groups (Table 6). Animals

were then inoculated with the lactate- and starch-utilizing bacteria (on day 0) and suddenly introduced to 800g/day/sheep of a 75% grain diet. According to the requirements of the Animal Ethics Committee, it was pre-determined that the animals would be withdrawn from the 75% barley diet to chaffed oat hay, if its rumen pH dropped below 5.0 or if the animal stopped eating for 24 hours. The animal would be further moved to the paddock and treated with 10% $NaHCO_3$ solution if more serious acidosis signs appeared.

Feed intake and diarrhoea score were measured daily. Animals were weighed on days -1, 4, and 11. Rumen fluid was collected for measuring pH, lactate, and VFA concentration on days -1, 2, 3, and 4. Faecal pH was also measured on day 4. Samples of blood were taken on days -1, 4 and 11 for measuring PCV.

Table 6 Experiment protocol

Day	Treatment group			
	Control	Su	Lu	Su+Lu
-19 to-1	D1	D1	D1	D1
0	D2	D2+SU	D2+LU	D2+SULU
1 to 11	D2	D2	D2	D2

D1, Chaffed oaten hay (800g/day/sheep). D2, 75% Grain pellet (800g/day/sheep) containing: Barley 75%, Chaffed lucerne hay 18%, Cottonseed meal 4.5%, Limestone 1.5%, Urea 1%, 0.1% Mineral salts. SU, 70 ml Su109 and 100 ml Su111 cultures. LU, 200 ml Lu12 culture. SULU, 70 ml Su109, 100 ml Su111, and 200 ml Lu12 cultures. The bacteria cultures were inoculated into the rumen through a stomach tube. The numbers of the bacteria cells used were 4.61 $\times 10^9$ /ml (Lu12), 5.89 $\times 10^9$ /ml (Su109), and 4.76 $\times 10^{10}$ /ml (Su111). Culture media are the same as those described in example 6.

Results

Withdrawn animals and feed intake

One animal from the Su group did not eat the 75% grain diet on days 0 and 1. This sheep was then given the chaff diet and ate well on the diet. The results from this animal were excluded from analysis. The number of animals, which were withdrawn from the 75% grain diet during the grain challenge period, is shown in Figure 8.

Six of the seven animals (85.7%) from the control group were withdrawn from the 75% grain diet during the experimental period. This ratio was the highest in the four groups. The group treated with a combination of lactate-utilizing and starch-fermenting bacteria had the lowest number of animals withdrawn from the grain diet over the first 8 days. After withdrawal from the 75% grain diet, two of the six control animals still did not eat on oat hay, became progressively sicker and could not stand up (lying down at all times). These two animals were returned to paddock grazing and treated with 10% $NaHCO_3$ solution on day 7. However, these animals did not recover and were humanely killed before the end of the trial. No animals treated with the starch-fermenting and/or lactate-utilizing bacteria were further withdrawn from the chaff to paddock (Table 7).

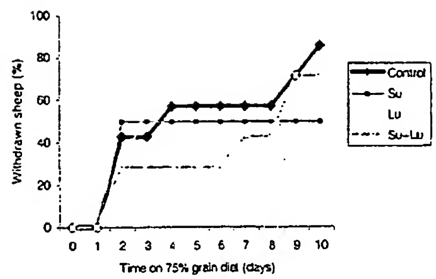


Figure 8 Number (%) of sheep withdrawn from the 75% grain diet during the period day 0 to 10. For the animal welfare and ethics reasons, it was pre-determined that any animal would be withdrawn from the 75% grain diet to chaffed oat hay, if its rumen pH dropped below 5.0 or it stopped eating for 24 hours

Statistical significant differences in feed intake between the treatment groups were not found on days 0 and 1 when all the animals were fed the 75% grain diet (P>0.05). During the period day 2 to 10, the mean feed intake of the Su+Lu animals on the 75% grain diet was significantly (P<0.05) higher than that of the control group (Table 7). There was no significant difference in the intake between the other groups (P>0.05). The mean intake of animals withdrawn and fed oat chaff in the Su+Lu group from day 2 to 10 was also significantly (P<0.01) higher than that of equivalent animals in the control group (Table 7). The intake of oat hay of Lu group was also significantly (P<0.05) higher than the control.

Table 7 Numbers of seriously sick animals and mean feed intake of sheep in different treatment groups during the period days 2 to 10

	Control	Su	Lu	Su+Lu
No of serious sick animals	2 †	0	0	0
Mean intake of 75% grain diet †† (g/sheep/day)	410 (63)	554 (58)	481 (63)	595 (48)*
Mean intake of oat hay (g/sheep/day) ††	346 (52)	432 (56)	517 (43)*	552 (60)**

†, After withdrawal from the 75% grain diet, the two animals still did not eat on oat hay, became progressively weaker.
††, The mean intake values were calculated on the total number of sheep on the 75% grain diet or on the oat hay over the whole period from day 2 to 10. Data presented in the bracket are the standard errors of the means by statistical analysis on this basis. *Significantly higher than the control (P<0.05) within the same row; ** highly significantly higher than the control (P<0.01) within the same row.

Severity of diarrhoea

Significant differences in diarrhoea scores of sheep in the different groups on the 75% grain diet were not found (P>0.05). In the animals fed on the oat hay, however, a significantly higher (P<0.05) diarrhoea score was observed in the control group on day 9, compared to the groups treated with lactate-utilizing and starch-fermenting bacteria (Figure 9).

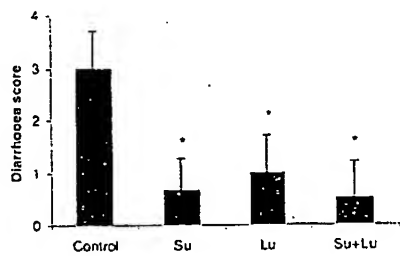


Figure 9 Mean diarrhoea score of sheep in different groups fed on the oat hay on Day 9. Vertical error bars represent standard errors of the means (least square means). *Significantly higher than the control (P<0.05)

PCV change and liveweight loss

Blood PCV change and liveweight loss of sheep in the different groups over the period of the experiment (day 0 to 11) are summarized in Table 8. The mean value of blood PCV in the control group increased by 1.5%, while that in the Su, Lu or Su+Lu groups decreased between 0.8 and 1.8%. The difference between the control and Su+Lu group was statistically significant (P<0.05). The liveweight loss in the control group was also significantly greater than that in the Su+Lu group (P<0.05).

Table 8 Mean blood PCV change (%) and liveweight loss (kg/sheep) of sheep in the different treatment groups during the period day 0 to 10 of all the animals. Data are presented as means ± SE

	Control	Su	Lu	Su+Lu
Blood PCV change (%)	1.5 ± 1.0	-0.8 ± 1.0	$-1.2 \pm ^{a} 0.9$	-1.8 ± 1.1*
Liveweight loss (kg)	-3.0 ± 0.5	-1.7 ± 0.5^{b}	-1.9 ± 0.4	-1.2 ± 0.5*

The values with "*" are significantly different from the control within the same row (P<0.05). The values with superscripts tend to be different from the control within the same row (^{a}P=0.07; ^{b}P=0.08)

Rumen pH, Lactate and VFA

The rumen pH in all the groups decreased significantly (P<0.01), while the lactate (L- and D-lactate) concentration increased 2 days following grain feeding (on day 2). On day 4 the rumen pH tended to increase, and the lactate concentration tended to decrease in all the treatment groups. The pH was highly associated with both L- and D-lactate concentration in the rumen fluid (r = -0.90, P<0.01). The rumen pH, L-lactate, D-lactate, and VFA concentration of sheep in the different treatment groups during the period day 0 to 4 are summarised in Figure 10. Significantly higher rumen pH and lower lactate (L- and D-lactate) concentrations were found in the Su+Lu group on day 4, compared with the control group (P<0.05). The VFA concentration tended to decrease in the control group, while it tended to increase in the Su, Lu and Su+Lu groups during day 0 to 4. The VFA concentrations in the Su, Lu and Su+Lu groups were also significantly higher than that in the control group on day 4. The proportions of individual acids in the total VFA of sheep fed 75% grain diet during the period day 2 to 4 are analysed. Compared with the control group, the Su+Lu group had lower acetic acid (P<0.05) and higher propionic acid (P<0.05) proportions (Liu, 1999).

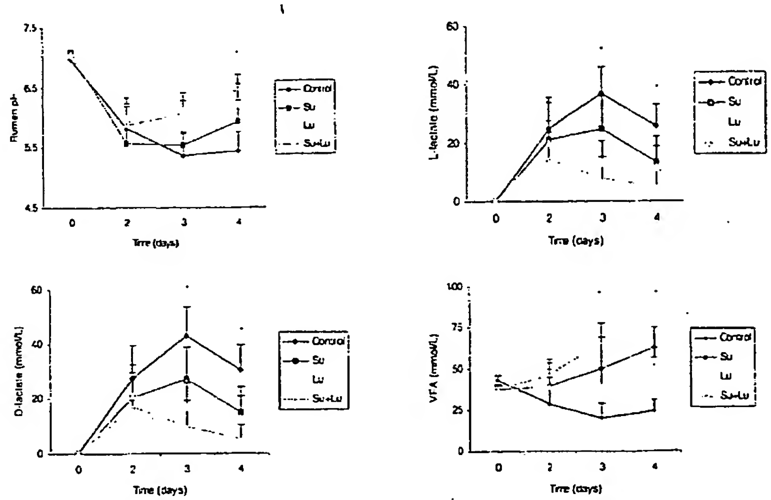


Figure 10 Mean rumen pH, L- and D-lactate, and VFA concentration (mmol/L) of sheep in different treatment groups during the period day 0 to 4. Vertical error bars represent standard errors of the means. *The rumen pH, L- and D-lactate, and VFA concentration in the Su+Lu group was significantly different from that in the control group (P<0.05); the rumen VFA concentrations in the Su, Lu, and Su+Lu groups were significantly higher than that in the control group on day 4 (P<0.05)

CONCLUSIONS AND DISCUSSION

When ruminants are fed diets rich in cereal grain, lactic acid-producing bacteria can overgrow and produce a large amount of lactic acid. The excessive accumulation of lactic acid in the rumen can cause a very low rumen pH and lead to lactic acidosis. One of the easily detectable and typical consequences of lactic acidosis is reduction of feed intake of the animal. Accordingly, rumen pH, lactate concentration, and feed intake of sheep were used as the key measurements to assess the efficacy of the lactate-utilizing and starch-fermenting bacteria for reducing the severity of lactic acidosis induced by a 75% grain diet in this experiment. For ethical and animal welfare reasons, it was critical to design an experimental protocol that could achieve the experimental objectives with the least suffering from lactic acidosis of sheep. According to previous experience, it was decided that a rumen pH below 5.0 or not eating for 24 hours due to the 75% grain challenge was the worst point that the animals could suffer from the grain challenge. In another words as described in the Materials and Methods section, the animals would be withdrawn from the 75% grain diet to chaffed oat hay, if rumen pH dropped below 5.0 or if the animal stopped eating for 24 hours due to the grain challenge. The animal would be further moved to the paddock and treated with 10% $NaHCO_3$ solution if more serious acidosis signs appeared.

Protection against lactic acidosis by Lu12, Su109 and Su111 was shown by fewer animals being withdrawn from the 75% grain challenge. At the final stage (on day 10), the control group had the most animals withdrawn from the 75% grain challenge (86%). Two of these withdrawn animals from the control group became very sick on the chaff diet and were further withdrawn to the paddock and treated with 10% $NaHCO_3$ solution on Day 7. Although from day 3 to 8, the Lu group had one more withdrawn animal than the control group, the Lu group had fewer withdrawn sheep than the control group over the whole period of grain challenge.

Statistically significant positive treatment effects were found with respect to feed intake, diarrhoea score, rumen pH, lactate, VFA, blood PCV and liveweight changes. These results are consistent with our previous work (Example 6) and indicate that Lu12, Su109 and Su111 can reduce the severity of lactic acidosis in animals rapidly introduced to a diet containing 75% grain. This experiment further demonstrated the potential for controlling lactic acidosis by lactate-utilizing and starch-fermenting bacterial strains Lu12, Su109 and Su111 in feedlot industry, in which the final ratio is normally around 75% grain (Feedlot Advisory Unit, 1990; ALFA, 1994).

Complete protection against acute lactic acidosis by the lactate-utilizing and starch-fermenting bacterial strains was not achieved under the present acute grain challenge conditions, in which the animals were suddenly introduced to a 75% well-processed grain pellet. This may be due to the dose of the probiotic bacteria not being great enough to protect the animals against the processed 75% barley pellet challenge. A better protection against lactic acidosis has been achieved using higher doses of Lu12, Su109 and/or Su111 and/or in conjunction with the grain-feeding management procedures.

Example 8 Improving starch utilisation and preventing acidosis in animals in other situations

Further studies also indicated that Lu12, Su109 and Su111 offered protection against lactic acidosis and improve the performance of sheep and cattle fed with diets rich in soluble carbohydrates under different feed regimes.

Example 9 Characteristics of the starch- and lactic acid-utilizing bacteria

To identify Lu12, Su109, and Su111, the system of bacterial classification and nomenclature used follow that presented in *Atlas of Rumen Microbiology* (Ogimoto and Imai, 1981) and *Bergey's Manual of Determinative Bacteriology* (Holt *et al.*, 1994). The classification of rumen bacteria is mainly based on the morphological, cultural, and metabolic features (Ogimoto and Imai, 1981). Accordingly, in this study several assays were carried out to observe the colony and growth appearance, motility, cell morphology and arrangement, Gram reaction, and size of these organisms. Substrate fermentation and metabolic characteristics of the strains were also studied. The 16s rRNA gene analyses were used to further identify the isolates Lu12, Su109 and Su111.

MATERIALS AND METHODS

For observing colony appearance, the culture of Lu12 was grown on MLH agar at 38.5 °C for about 48 hours. The colony appearance of Su109 and Su111 was observed on mRF agar after incubation for approximately 48 hours. Cultures of Lu12 in MLH broth, Su109 and Su111 in mRF broth were used for observing the growth appearance, motility, morphology, Gram reaction, and size. Motility was measured by observing the movement of bacterial cells under an Olympus microscope using oil immersion. Shape and size of the bacterial cells were observed using oil immersion and electronic microscopy (Ogimoto and Imai, 1981; Holt *et al.*, 1994).

Basic medium (Liu, 1999) was used for preparing the substrate fermentation media. Twenty-eight substrates (Table 10) were selected and used in this experiment according to the method described by Ogimoto and Imai (1981). The methods of 16s rRNA gene analyses used to further identify the isolates Lu12, Su109 and Su111 were described by Liu (1999).

RESULTS

The growth appearance, morphology, and Gram reaction are described in Table 9. All three strains exhibited different colony and growth appearance. The lactate-utilizing strain Lu12 had motility while the other two starch-fermenting bacteria (Su109 and Su111) were non-motile strains. The morphology and cell arrangements of the three strains were completely different. However, all three strains were Gram-negative bacteria.

The three strains can all ferment starch, but only Lu12 can utilize lactate. Table 10 shows the substrate fermentation of the three strains. The fermentation patterns of the three strains are remarkably different.

The results of the 16S rDNA sequence analysis of the strains are summarised as follows (see page 26). Phylogenetic analysis (Table 11) suggests that Lu12 may be a newly isolated strain of *Selenomonas ruminantium* (with at least 0.99 similarity to the four *Selenomonas ruminantium* reference strains), while Su 111 appears to be a new species of *Prevotella* (with 0.88-0.92 similarity to the 13 reference *Prevotella* organisms). Su109 had low similarities (0.85-0.87) to the 13 reference *Prevotella* organisms, while it had even lower similarities (0.71-0.76) to the other 24 pure strains from several different genera (Table 11). However, Su109 had 0.89 and 0.91 similarities to the reference rumen clones RFN2 and 12-129 (from a big rumen clone library), respectively.

CONCLUSIONS AND DISCUSSION

The characteristics of Lu12, Su109, and Su111 investigated in this study do not completely match any strains described in *Bergey's Manual of Determinative Bacteriology* (Holt *et al.*, 1994), *Bergey's Manual of Systematic Bacteriology* (Krieg *et al.*, 1984), or *Atlas of Rumen Microbiology* (Ogimoto and Imai, 1981).

According to the bacteriological identification criteria described by Ogimoto and Imai (1981), Krieg *et al.* (1984) and Holt *et al.* (1994), Lu12 is similar to *Selenomonas ruminatium* subsp. *lactilytica*. However, the substrate fermentation patterns of Lu12 are different to the strains described by Ogimoto and Imai (1981), Krieg *et al.* (1984) and Holt *et al.* (1994). Lu12 did not ferment amygdalin, dulcitol, fructose, glycerol, or inulin. The 16S rDNA's sequence and phylogenetic analysis further confirmed that Lu12 is a newly isolated *Selenomonas ruminatium* strain (namely, *Selenomonas ruminatium* subsp. *lactilytica* Lu12).

Su111 has some similar biological characteristics to the strains of *Prevotella ruminicola*. However, we observed that Su111 did not ferment amygdalin and galactose. Phylogenetic analysis demonstrated that Su111 was closest to the *Prevotella* species but with only 0.88-0.92 similarity in 16S rDNA's sequence of 13 reference *Prevotella* organisms. This suggests that Su111 is suitable to be classified as a new *Prevotella* spp. (namely, *Prevotella shuii* Su111). The new specific name was given following the recommendations described by Lapage *et al.*, 1992.

The biological characteristics of Su109 are similar to those of *Bacteriodes eggerthii*, which has been isolated from human faeces (Krieg *et al.*, 1984). However, unlike *Bacteriodes eggerthii*, Su109 fermented melibiose and sucrose. Phylogenetic analysis further indicated that Su109 had low similarities to those pure cultures (0.85-0.87 to *Prevotella* spp. and 0.71-0.76 to the other pure organisms from several different genera) but had 0.89 to 0.91 similarity of 16S rDNA's sequence to the reference rumen clones RFN2 and 12-129 from a big rumen clone library. These facts suggest that it may be appropriate to classify Su109 as a new genus (namely, *Shuia liuiae* Su109). The new generic name was given following the recommendations described by Lapage *et al.*, 1992.

Table 9 Colony appearance, growth, motility, morphology, Gram reaction, and size of Lu12, Su109, and Su111

Observation	Characteristics		
	Lu12	Su109	Su111
Colony appearance	Translucent to opaque, Grey to white, smooth, and round colony	Grey to white, convex, and smooth colony	Grey to white and smooth colony
Appearance of culture broth	Light yellow to white colour	White and turbid with much white precipitate	White and turbid in mRF broth with some white precipitate
Motility	Yes	No	No
Morphology	Curve rods, round ends, crescent-shaped cells. Arranged singly or in pairs, occasionally in short chain and clumps	Straight rods with round ends, occur singly, in pairs, and occasionally arranged as letter (x, v, y, T) shapes. Slightly curved rods and spindle ends were observed occasionally	Coccoid to oval or short rod-shaped. Arranged in pairs or short chain, occasionally occur singly
Gram reaction	Negative	Negative	Negative
Size	0.4-0.8 by 1.7-3.0 µm	0.3-0.6 by 1.2-6.0 µm	0.4 –0.8 by 0.7 –2.6 µm

Table 10 Fermentation of substrates by Lu12, Su109, and Su111

Substrates	Lu12	Su109	Su111
Adonitol	-	-	-
Amygdalin	-	±	-
Arabinose	+	+	+
Cellobiose	+	+	+
Dextrin	-	+	+
Dulcitol	-	-	-
Erythritol	-	-	-
Esculin	+	-	+
Fructose	-	+	+
Galactose	+	+	-
Glucose	+	+	+
Glycerol	-	-	-
Glycogen	-	+	+
Inulin	-	-	+
Lactose	+	+	+
Maltose	+	+	+
Mannitol	+	-	-
Mannose	+	+	+
Melibiose	+	+	-
Raffinose	+	-	+
Rhamnose	-	+	+
Ribose	+	-	-
Salicin	+	-	-
Sorbitol	-	-	-
Starch	+	+	+
Sucrose	+	+	+
Xylose	+	+	+
Lactate	+	-	-

Positive fermentation (+) was determined on the basis of pH change and/or visible growth (turbidity) in the broth after 24-48 hours incubation. "±", Slight turbidity in the broth after incubation was observed but the pH remained the same as the blank control.

Partial sequence of 16S rDNA of isolate Lu12 (655 bp):

```
  1  GGCGAGCGTT GTCCGGAATT ATTGGGCGTA AAGGGAGCGC AGGCGGGAAG
 51  GCAAGTCAGT CTTAAAAGTG CGGGGCTCAA CCCCGTGATG GGATTGAAAC
101  TGTCTTTCTT GAGTGCAGGA GAGGAAAGCG GAATTCCTAG TGTAGCGGTG
151  AAATGCGTAG ATATTAGGAG GAACACCAGT GGCGAAGGCG GCTTTCTGGA
201  CTGTAACTGA CGCTGAGGCT CGAAAGCGTG GGGAGCGAAC AGGATTAGAT
251  ACCCTGGTAG TCCACGCCGT AAACGATGAA TGCTAGGTGT AGGAGGTATC
301  GACCCCTTCT GTGCCGGAGT TAACGCAATA AGCATTCCGC CTGGGGAGTA
351  CGGTCGCAAG ACTGAAACTC AAAGGAATTG ACGGGGGCCC GCACAAGCGG
401  TGGAGTATGT GGTTTAATTC GACGCAACGC GAAGAACCTT ACCAGGGCTT
451  GACATTGAGT GAAAGATCTA GAGATAGATC CCTCTCTTCG GAGACACGAA
501  AACAGGTGGT GCATGGCTGT CGTCAGCTCG TGTCGTGAGA TGTTGGGTTA
551  AGTCCCGCAA CGAGCGCAAC CCCTATCATT TGTTGCCAGC ACGTTAAGGT
601  GGGAACTCAA ATGAGACTGC CGCGGACAAC GCGGAGGAAG GCGGGGATGA
651  CGTCA
```

Partial sequence of 16S rDNA of isolate Su1111 (575 bp):

```
  1  CGGATTTATT GGGTTTAAAG GGAGCGCAGG CCGTTTGGTA AGCGTGTTGT
 51  GAAATGTCCG GGCTCAACCT GGGCACTGCA GCGCGAACTG TCAGACTTGA
101  GTGCACAGGA AGCGGGCGGA ATTCGTGGTG TAGCGGTGAA ATGCTTAGAT
151  ATCACGAAGA ACTCCAATTG CGAAGGCAGC TCGCTGTAGT GTTACTGACG
201  CTAAAGCTCG AAAGTGCGGG TATCGAACAG GATTAGATAC CCTGGTAGTC
251  CGCACGGTAA ACGATGGATG CCCGCTGTTT GCCCTTCGGG GTGAGTGGCT
301  AAGCGAAAGC GTTAAGCATC CCACCTGGGG AGTACGCCGG CAACGGTGAA
351  ACTCAAAGGA ATTGACGGGG GCCCGCACAA GCGGAGGAAC ATGTGGTTTA
401  ATTCGATGAT ACGCGAGGAA CCTTACCCGG GCTTGAATTG CAGATGACGG
451  ATCTAGAGAT AGAGACTTCC TTCGGGACAT CTGTGAAGGT GCTGCATGGT
501  TGTCGTCAGC TCGTGCCGTG AGGTGTCGGC TTAAGTGCCA TAACGAGCGC
551  AACCCTTCTC TTCAGTTGCC ATCAG
```

Full sequence of 16S rDNA of isolate Su109 (1450 bp):

```
   1  GATGAACGCT AGCTACAGGC TTAACACATG CAAGTCGAGG GGCAGCATTA
  51  AGTCAGCTTG CTGATTTAGA TGGCGACCGG CGCACGGGTG AGTAACGCGT
 101  ATCCAACCTG CCCCCTACCC GGGGATAGCC TTGCGAAAGT AAGATTAATA
 151  CCCGGTGCTG TTATGATTCC GCATGGGAAT ATAACGAAAG ATTCATCGGT
 201  AGGGGATGGG GATGCGTCCG ATTAGCTTGT TGGCGGGGTA ACGGCCCACC
 251  AAGGCGACGA TCGGTAGGGG TTCTGAGAGG AAGGTCCCCC ACATTGGAAC
 301  TGAGACACGG TCCAAACTCC TACGGGAGGC AGCAGTGAGG AATATTGGTC
 351  AATGGGCGAG AGCCTGAACC AGCCAAGTAG CGTGAAGGAA GACTGCCCTA
 401  CGGGTTGTAA ACTTCTTTTG TACGGGAATA AAGTGTGCCA CGTGTGGCGT
 451  TTTGCATGTA CCGTACGAAT AAGGACCGGC TAATTCCGTG CCAGCAGCCG
 501  CGGTAATACG GAAGGTCCGA GCGTTATCCG GATTTATTGG GTTTAAAGGG
 551  AGCGCAGGCG GAATGTTAAG TCAGCTGTGA AATCCCGTCG CTCAACGGCG
 601  GAACTGCAGT TGATACTGGC TTTCTTGAGT GCACATAAGG ATGGTGGAAT
 651  TCGTGGTGTA GCGGTGAAAT GCTTAGATAT CACGAAGAAC TCCGATTGCG
 701  AAGGCAGCCA TCTGGGGTGC AACTGACGCT GAGGCTCGAA AGTGCGGGTA
 751  TCAAACAGGA TTAGATACCC TGGTAGTCCG CACAGTAAAC GATGAATACT
 801  CGCTGTCGGC GATATACAGT CGGCGGCCAA GCGAAAGCAT TAAGTATTCC
 851  ACCTGGGGAG TACGCCGGCA ACGGTGAAAC TCAAAGGAAT TGACGGGGGC
 901  CCGCACAAGC GGAGGAACAT GTGGTTTAAT TCGATGATAC GCGAGGAACC
 951  TTACCCGGGC TTGAACTGCA CCCGACGGAC AGAGAGATTT GTCTTCCGCA
1001  AGGCGGGTGT GGAGGTGCTG CATGGTTGTC GTCAGCTCGT GCCGTGAGGT
1051  GTCGGCTTAA GTGCCATAAC GAGCGCAACC CTTCTCTTCA GTTGCCATCA
1101  GGTAGAGCTG GGCACTCTGG AGACACTGCC ATCGTAAGAT GCGAGGAAGG
1151  TGGGGATGAC GTCAAATCAG CACGGCCCTT ACGTCCGGGG CTACACACGT
1201  GTTACAATGG GAGGTACAGA AGGCYGCGAC CCGGCGACGG GAAGCCAATC
1251  CCCAAATCCT CTCTCAGTTC GGACTGGAGT CTGCAACCCG ACTCCACGAA
1301  GCTGGATTCG CTAGTAATCG CGCATCAGCC ATGGCGCGGT GAATACGTTC
1351  CCGGGCCTTG TACACACCGC CCGTCAAGCC ATGAAAGCCG GGGTACCTG
1401  AAGTCCGTAA CCGCAAGGAG CGGCCTAGGG TAAAACTGGT AATTGGGGCT
```

Table 11 . Similarity matrix of Lu12, Su109, Su111 and reference rumen bacteria based on comparisons of 16S rDNA sequences

		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35	S36	S37	S38	S39
PreNigre	S1
PreInte2	S2	0.94
PreDisie	S3	0.92	0.94
PreCorpo	S4	0.93	0.93	0.92
PreBivia	S5	0.90	0.91	0.92	0.91
PreOris	S6	0.90	0.90	0.89	0.90	0.91
PreOulue	S7	0.91	0.91	0.93	0.92	0.92	0.93
PreMela2	S8	0.92	0.92	0.92	0.91	0.93	0.91	0.91
PreVeros	S9	0.91	0.91	0.91	0.92	0.93	0.91	0.91	0.97
PreDent2	S10	0.91	0.90	0.91	0.91	0.89	0.91	0.91	0.94	0.93
PreBucc2	S11	0.88	0.87	0.87	0.89	0.88	0.89	0.89	0.88	0.89	0.91
PreOrali	S12	0.88	0.87	0.87	0.88	0.89	0.89	0.89	0.89	0.88	0.88	0.89
PreRumin	S13	0.87	0.88	0.88	0.88	0.90	0.88	0.89	0.89	0.89	0.88	0.87	0.88
LacSaliv	S14	0.72	0.72	0.73	0.73	0.73	0.73	0.73	0.73	0.72	0.72	0.71	0.72	0.71
LacAviar	S15	0.72	0.71	0.73	0.72	0.72	0.72	0.73	0.73	0.73	0.72	0.71	0.72	0.72	0.95
LacAgili	S16	0.72	0.71	0.72	0.72	0.72	0.73	0.73	0.72	0.71	0.71	0.71	0.72	0.72	0.94	0.94
LacRumin	S17	0.73	0.72	0.73	0.73	0.73	0.72	0.74	0.73	0.73	0.72	0.71	0.72	0.73	0.95	0.93	0.93
LacAnima	S18	0.73	0.72	0.73	0.73	0.73	0.72	0.74	0.73	0.73	0.72	0.71	0.72	0.73	0.94	0.93	0.94	0.94
LacMurin	S19	0.72	0.72	0.72	0.72	0.72	0.72	0.73	0.73	0.72	0.72	0.70	0.72	0.72	0.94	0.93	0.96	0.94	1.00
LacMali	S20	0.72	0.72	0.72	0.72	0.73	0.73	0.73	0.73	0.72	0.72	0.71	0.72	0.72	0.94	0.94	0.94	0.94	0.95	0.95
LacCase4	S21	0.72	0.73	0.72	0.72	0.72	0.72	0.73	0.73	0.72	0.72	0.71	0.73	0.72	0.91	0.91	0.91	0.89	0.91	0.91	0.92
CloSpiro	S22	0.71	0.70	0.70	0.71	0.70	0.71	0.72	0.72	0.71	0.71	0.70	0.69	0.71	0.82	0.82	0.82	0.82	0.83	0.83	0.82	0.83
CloRamos	S23	0.71	0.71	0.70	0.72	0.71	0.72	0.71	0.72	0.71	0.71	0.70	0.69	0.71	0.82	0.82	0.83	0.83	0.84	0.83	0.83	0.81	0.98
LacVitul	S24	0.72	0.71	0.70	0.71	0.69	0.72	0.73	0.71	0.71	0.72	0.72	0.70	0.70	0.80	0.80	0.80	0.81	0.81	0.81	0.81	0.81	0.83	0.83
LacCaten	S25	0.72	0.71	0.71	0.71	0.70	0.72	0.73	0.72	0.71	0.72	0.71	0.70	0.70	0.79	0.80	0.80	0.79	0.80	0.80	0.80	0.81	0.84	0.83	0.90
SelRumi2	S26	0.72	0.73	0.72	0.72	0.73	0.72	0.74	0.73	0.72	0.71	0.72	0.71	0.73	0.82	0.82	0.82	0.82	0.83	0.83	0.82	0.82	0.79	0.79	0.78	0.79
SrGA192	S27	0.72	0.73	0.72	0.72	0.74	0.71	0.74	0.71	0.72	0.73	0.72	0.71	0.73	0.82	0.82	0.82	0.82	0.83	0.82	0.82	0.82	0.79	0.80	0.79	0.79	0.99
SelRumi4	S28	0.72	0.72	0.72	0.73	0.73	0.73	0.74	0.73	0.72	0.73	0.72	0.74	0.72	0.82	0.82	0.83	0.83	0.83	0.82	0.82	0.82	0.80	0.80	0.79	0.78	0.98	0.98
SelLact2	S29	0.72	0.72	0.72	0.71	0.72	0.72	0.73	0.71	0.72	0.72	0.71	0.70	0.73	0.83	0.82	0.82	0.81	0.83	0.82	0.81	0.82	0.80	0.83	0.79	0.78	0.94	0.94	0.91
SelSputi	S30	0.71	0.72	0.71	0.71	0.71	0.72	0.73	0.71	0.72	0.72	0.72	0.71	0.74	0.82	0.81	0.81	0.81	0.81	0.80	0.80	0.80	0.78	0.79	0.80	0.79	0.91	0.90	0.92	0.91
QuiOvali	S31	0.71	0.71	0.71	0.71	0.70	0.71	0.72	0.71	0.70	0.71	0.70	0.70	0.10	0.81	0.81	0.80	0.81	0.81	0.81	0.82	0.80	0.78	0.78	0.78	0.78	0.86	0.86	0.87	0.86	0.86
PecFrisi	S32	0.72	0.73	0.72	0.72	0.72	0.73	0.74	0.73	0.72	0.72	0.72	0.73	0.73	0.81	0.81	0.80	0.80	0.81	0.80	0.81	0.80	0.78	0.79	0.79	0.79	0.88	0.89	0.89	0.89	0.90	0.85
12-129	S33	0.86	0.86	0.85	0.85	0.86	0.86	0.87	0.86	0.86	0.86	0.85	0.85	0.84	0.75	0.72	0.73	0.74	0.73	0.72	0.75	0.73	0.71	0.72	0.70	0.70	0.74	0.74	0.74	0.73	0.72	0.73	0.72
SrS20	S34	0.71	0.72	0.71	0.71	0.72	0.72	0.73	0.72	0.71	0.72	0.71	0.70	0.72	0.81	0.81	0.82	0.82	0.82	0.82	0.81	0.81	0.79	0.79	0.78	0.78	0.90	0.90	0.88	0.91	0.91	0.86	0.88	0.73
MitMult	S35	0.72	0.72	0.72	0.72	0.72	0.73	0.74	0.73	0.72	0.73	0.74	0.71	0.72	0.83	0.81	0.81	0.81	0.82	0.82	0.81	0.81	0.79	0.80	0.79	0.78	0.95	0.93	0.95	0.93	0.91	0.86	0.89	0.73	0.93
SrJCM6582	S36	0.71	0.72	0.72	0.72	0.71	0.72	0.73	0.72	0.71	0.72	0.71	0.70	0.72	0.82	0.81	0.82	0.82	0.82	0.82	0.82	0.81	0.78	0.79	0.79	0.70	0.98	0.90	0.98	0.94	0.91	0.86	0.88	0.73	0.90	0.99
Su109	S37	0.86	0.86	0.85	0.87	0.86	0.86	0.87	0.87	0.87	0.86	0.86	0.85	0.86	0.73	0.73	0.74	0.74	0.74	0.73	0.73	0.78	0.72	0.73	0.72	0.71	0.75	0.75	0.75	0.74	0.72	0.72	0.73	0.80	0.74	0.74	0.75
Lu12	S38	0.72	0.74	0.74	0.74	0.75	0.75	0.74	0.74	0.73	0.73	0.72	0.73	0.74	0.93	0.94	0.85	0.84	0.85	0.84	0.84	0.84	0.80	0.82	0.81	0.78	1.00	1.00	0.99	0.96	0.95	0.86	0.92	0.73	1.00	0.97	1.00	0.76
Su111	S39	0.88	0.90	0.90	0.90	0.92	0.89	0.88	0.89	0.90	0.88	0.89	0.90	0.89	0.73	0.73	0.76	0.76	0.73	0.76	0.73	0.76	0.69	0.70	0.68	0.69	0.73	0.75	0.76	0.74	0.74	0.73	0.76	0.66	0.73	0.73	0.76	0.66	0.76
RFN2	S40	0.84	0.84	0.85	0.85	0.85	0.86	0.87	0.88	0.86	0.85	0.84	0.83	0.85	0.72	0.71	0.73	0.73	0.71	0.72	0.72	0.73	0.71	0.72	0.71	0.70	0.73	0.73	0.73	0.71	0.71	0.71	0.73	0.83	0.73	0.73	0.73	0.91	0.76	0.84

PreNigre, *Prevotella nigrescens*, L16479, 1465 , 1
PreInte2, *Prevotella intermedia*, L16468, 1470 , 0
PreDisie, *Prevotella disiens*, L16483, 1479 , 0
PreCorpo, *Prevotella corporis*, L16465, 1473 , 0
PreBivia, *Prevotella bivia*, L16475, 1471 , 0
PreOris, *Prevotella oris*, L16474, 1478 , 0
PreOulue, *Prevotella oulora*, L16472, 1473 , 3
PreMela2, *Prevotella melaninogenica*, L16469, 1478 , 1
PreVeros, *Prevotella veroralis*, L16473, 1478 , 0
PreDent2, *Prevotella denticola*, L16466, 1471 , 0
PreBucc2, *Prevotella buccae*, L16478, 1474 , 0

PreRumin, *Prevotella ruminicola*, L16482, 1462 , 1
PreOrali, *Prevotella oralis*, L16480, 1481 , 1
Su111, 575 , 0
12-129, rumen clone 12-129, AF018497, 1449 , 0

Su109, 1450 , 0
RFN2, rumen clone RFN2, AF018778, 1423 , 0
SelRumi2, *Selenomonas ruminantium* str.HD4, M62703, 1380 , 0
Lu12, 655 , 0
SrJCM6582, *Selenomonas ruminantium* str.JCM6582, AB003379, 1455 , 0
SrS20, *Selenomonas ruminantium* str.S20, AB017195, 1475 , 0

SrGA192, *Selenomonas ruminantium* str.GA192, M62702, 1479 , 0
SelRumi4, *Selenomonas ruminantium*, 1451 , 0
MitMult, *Mitsuokella multiacidus*, X81878, 1511 , 0
SelLact2, *Selenomonas lacticifex*, 1456 , 0
SelSputi, *Selenomonas sputigena*, 1499 , 0
PecFrisi, *Pectinatus frisingensis*, 1440 , 0
QuiOvali, *Quinella ovalis*, M62701, 1470 , 1
LacSaliv, *Lactobacillus salivarius*, M59054, 1512 , 0
LacAviar, *Lactobacillus aviarius*, M58808, 1505 , 1
LacAgili, *Lactobacillus agilis*, M58803, 1508 , 1
LacAnima, *Lactobacillus animalis*, M58807, 1504 , 2

LacMurin, *Lactobacillus murinus*, M58826, 1492 , 1
LacMali, *Lactobacillus mali*, M58824, 1501 , 1
LacRumin, *Lactobacillus ruminis*, M58828, 1542 , 0
LacCase4, *Lactobacillus casei*, M23928, 1548 , 0
CloSpiro, *Clostridium spiroforme*, X73441, 1511 , 0
CloRamos, *Clostridium ramosum*, X73440, 1519 , 0
LacCaten, "*Lactobacillus*" catenaformis, M23729, 1515 , 1
LacVitul, "*Lactobacillus*" vitulinus, M23727, 1477 , 1

Claims

1. Methods for improving starch utilization and preventing over-production of acid in the rumen or gut of animals (e.g. cattle, sheep, goat, horses, and other animals) and/or reducing the adverse effects due to the over-production of acid (e.g. acidosis and relevant adverse effects) in animals, comprising the administration to the animals of one or more of the following strains of starch-utilizing bacteria and/or lactate-utilizing bacteria:
   (1) Starch-utilizing bacteria strains Su109 (*Shuia liuiae* Su109), Su111 (*Prevotella shuii* Su111), and their mutant(s)/variant(s).
   (2) Lactate-utilizing bacteria strains Lu12 (*Selenomonas ruminatium* subsp. *lactilytica* Lu12), and its mutant(s)/variant(s).

2. Probiotics for improving starch utilization and preventing over-production of acid in the rumen or gut of animals (e.g. cattle, sheep, goat, horses, and other animals) and/or reducing the adverse effects due to the over-production of acid (e.g. acidosis and relevant adverse effects) in animals, comprising the administration to the animals of one or more of the following strains of starch-utilizing bacteria and/or lactate-utilizing bacteria:
   (1) Starch-utilizing bacteria strains Su109 (*Shuia liuiae* Su109), Su111 (*Prevotella shuii* Su111), and their mutant(s)/variant(s).
   (2) Lactate-utilizing bacteria strains Lu12 (*Selenomonas ruminatium* subsp. *lactilytica* Lu12), and its mutant(s)/variant(s).

3. The methods and probiotics according to claims 1 and 2 comprising whole cells, cell lysatse, enzymes, any part of the cells, or any product from the microorganism(s).

4. The methods and probiotics according to any one of claims 1 to 3 further including pharmaceutically or commercially acceptable carriers or excipients.

5. Organism(s), or relevant part of the organism(s), or their product(s) of claims 1 to 4 in viable form, lyophilised form, or any other forms.

6. A composition comprising any of the above organism(s) or any part of the organism(s) or any product(s) of the organism(s) of any one of claims 1 to 5 in a carrier material.

7. A feed or feed additive containing any of the strain(s), or any part of the cells of the strain(s), or any product(s) of the organism(s) of the claims 1 to 6.

8. The cells, or any part of the cells, or any product(s) of the cells of the organism(s) according to any one of the claims 1 to 6.

Quan Shu, Aihua Liu, and Dragon Pacific Limited
6 May 2002

on behalf of Dragon Pacific Ltd